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GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF PLASMA LEVELS OF *d*-7,8-DIMETHOXY-3-METHYL-PHENYL-2,3,4,5-TETRAHYDRO-1H-3-BENZAZEPINE ACID MALEATE (SCH-12679) AND ITS MAJOR METABOLITES IN AGGRESSIVE MENTAL RETARDATES

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SUMMARY

A sensitive gas—liquid chromatographic technique for the quantitative analysis of SCH-12679 (*d*-7,8-dimethoxy-3-methyl-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine acid maleate) and its major metabolites in plasma of aggressive mental retardates receiving therapeutic doses of the medicament has been developed. The lower limits of detection are 20 ng/ml for SCH-12679, 0.5 ng/ml for 3-desmethyl SCH-12679 and 0.4 ng/ml for 7-desmethyl plus 8-desmethyl SCH-12679. SCH-12679 is estimated with a flame ionization detector. Its metabolites are quantitated using an electron-capture detector after conversion of the compounds to their heptafluorobutyryl derivatives by reaction with the ap-

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propriate anhydride. Data on plasma levels of unchanged SCH-12679, 3-desmethyl SCH-12679 and a combination of 7-desmethyl and 8-desmethyl SCH-12679 in fifteen patients treated with the medicament are presented.

INTRODUCTION

SCH-12679 (*d*-7,8-dimethoxy-3-methyl-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine acid maleate) is a novel chemical entity (Fig. 1A) with an interesting pharmacological profile. It exhibits a broad spectrum of activity in both laboratory animals [1] and humans [2—6]. Recently we have reported that SCH-12679 is an effective drug in the treatment of aggressive mental retardates [7]. The objective of this study was to develop a sensitive, selective, gas–liquid chromatographic (GLC) method for the quantification of unchanged SCH-12679 and its major metabolites (Fig. 1B, C and D) in plasma of mentally retarded patients after therapeutic doses of this medicament.

MATERIALS AND METHODS

SCH-12679, 3-desmethyl SCH-12679, 7-desmethyl SCH-12679 and 8-desmethyl SCH-12679 for chromatographic standards, as well as SCH-12679 capsules for oral administration to patients were supplied by Schering (Bloomfield, N.J., U.S.A.). Protriptyline was used as an internal standard for GLC analysis.

Reagents and materials

The following organic reagents were used: GLC—spectrometric quality

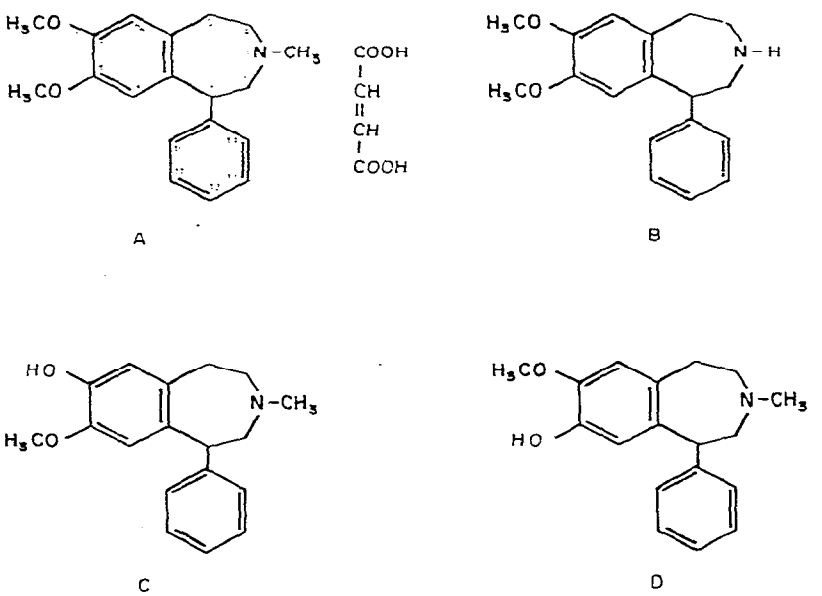

Fig. 1. Structural formulae of SCH-12679. (A) SCH-12679 maleate; (B) 3-desmethyl SCH-12679; (C) 7-desmethyl SCH-12679; (D) 8-desmethyl SCH-12679.

toluene and methanol (J.T. Baker, Phillipsburg, N.J., U.S.A.), MC/B "chromatoquality" isoamyl alcohol (Matheson Coleman and Bell, East Rutherford, N.J., U.S.A.), heptafluorobutyric anhydride (HFBA) (Eastman Kodak, Rochester, N.Y., U.S.A.) and β-glucuronidase type H-2 (Sigma, St. Louis, Mo., U.S.A.). The inorganic reagents were made up in doubly distilled water. The Dubnoff metabolic shaking incubator was purchased from Fisher Scientific (Montreal, Canada).

Gas—liquid chromatography

Analyses were performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with dual flame ionization detectors (FID) and a ^{63}Ni (15 mCi) electron-capture detector (ECD). The apparatus had automation capability built into its digital processor (HP 18850 A) to operate the gas chromatograph throughout the analytical run, following precisely the instructions given on its keyboard. The processor continuously monitored detector output and reduced it to peak areas and retention times.

The chromatographic conditions for SCH-12679 and its major metabolites were as follows.

SCH-12679. A 1.8-m coiled glass column (O.D. 6.3 mm, I.D. 4 mm) was packed with 3% PC 3210 (50% SE-30 ultraphase + 50% OV-210) on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties, Brockville, Canada). The column was conditioned at 275° for 24 h with a nitrogen carrier gas flow-rate of 45 ml/min. The column was operated at 200°, the FID at 260° and the injection port at 250° with the following gas flow-rates: nitrogen 45 ml/min, hydrogen 55 ml/min and air 180 ml/min. Under these conditions, the relative retention time of SCH-12679 to the internal standard (protriptyline) was 1.23 (Fig. 2B).

Major metabolites of SCH-12679. A 1.8-m coiled glass column, (O.D. 6.3 mm, I.D. 4 mm) was packed with 3% Apiezon L on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties). The column was conditioned at 275° for 24 h with an argon—methane (95:5) carrier flow-rate of 50 ml/min. The column was operated at 220°, the ECD at 300° and the injection port at 250° with carrier flowing at 50 ml/min. The two isomers, namely 7-desmethyl and 8-desmethyl SCH-12679, could not be separated under the present GLC conditions. Therefore the assay yields the sum of both. The relative retention times of the isomers and 3-desmethyl SCH-12679 to the internal standard (protriptyline) were 0.416 and 0.814, respectively (Fig. 3B).

Hydrolysis of the conjugated compounds

In order to hydrolyse the conjugated metabolites of SCH-12679, pooled plasma of the patients who were treated with this drug was processed by the following methods.

(A) To 10 ml of pooled plasma was added 0.5 ml of β-glucuronidase type H-2. The mixture was adjusted to pH 5 with 0.1 *M* sodium acetate buffer and incubated at 37° for 24 h in a Dubnoff metabolic shaking incubator. After cooling at room temperature the mixture was brought to pH 10 with 10 *N* NaOH and extracted as described in the extraction procedure. It was found

that the hydrolysis was incomplete and the plasma extract gave several extraneous background peaks, which could interfere with the assay.

(B) To 10 ml of pooled plasma were added 10 ml of 1 *N* HCl and the mixture was heated at 95° for 30 min. After cooling at room temperature, the mixture was adjusted to pH 10 with 10 *N* NaOH and extracted according to the extraction procedure. It was noticed that substantial degradation of the parent drug and its metabolites occurred using the acid hydrolysis process.

(C) To 10 ml of pooled plasma were added 8 ml of 10 *N* NaOH and the mixture was heated at 95° for 30 min. After cooling at room temperature 3.5 ml of water were then added to the mixture, which was later extracted in the same way. It was observed that the hydrolysis was more effective without chemical degradation of the parent drug and its metabolites. This observation was confirmed by comparing the quantities of SCH-12679 and its metabolites in 10 ml of non-hydrolysed pooled plasma of the patients (same lot) with the hydrolysed sample. The quantity of 7-desmethyl and 8-desmethyl SCH-12679 was approximately three times more in the hydrolysed than in the non-hydrolysed pooled plasma. Huang and Kurland [8] also suggested that alkaline hydrolysis is preferable to acid hydrolysis for the phenothiazine compounds. For the hydrolysis of conjugated metabolites the alkaline hydrolysis was adopted in this study.

Extraction procedure

To 3 ml of plasma in a 13-ml glass-stoppered centrifuge tube were added 2.4 ml of 10 *N* NaOH. The mixture was heated at 95° for 30 min on an electric plate to hydrolyse the conjugated metabolites. The mixture was allowed to cool at room temperature. One milliliter of water was then added to the mixture, which was later totally transferred to a 50-ml centrifuge tube.

A 25-μl volume of the methanolic solution of the internal standard, protriptyline hydrochloride (100 μg/ml), were added to the mixture, which was then extracted twice with 5 ml of toluene containing 1.5% isoamyl alcohol. After each extraction the tubes were agitated in a mechanical shaker for 15 min and then centrifuged at 600 *g* for 10 min. The organic layer was transferred to a 13-ml centrifuge tube and the aqueous layer was discarded. The organic layer was extracted with 2 ml of 0.1 *M* phosphate buffer (pH 7.2) for 15 min and centrifuged for 10 min. The buffer layer was preserved for further extraction. The organic layer was back-extracted with 2 ml of 0.05 *N* HCl for 15 min. The sample was centrifuged as before and the organic layer was removed and discarded. The aqueous layer was made alkaline with 0.2 ml of 1 *N* NH_4OH solution and extracted with 1.5 ml of toluene—isoamyl alcohol mixture (98.5:1.5) for 15 min, then centrifuged as described above. The organic layer was transferred to a 5-ml conical glass-stoppered tube (A).

The phosphate buffer layer from the previous extraction was made alkaline with 0.2 ml of 1 *N* NH_4OH solution and extracted with 1.5 ml of toluene—isoamyl alcohol mixture (98.5:1.5) for 15 min. After centrifugation, the organic layer was transferred to the previous tube (A) and evaporated to dryness at 45° with a low stream of nitrogen. The aqueous layer was discarded. The walls of the tube were rinsed with 0.1 ml of methanol by vibrating with

a Vortex mixer for 1 min. The solution was evaporated to dryness as before and the residue was used further for quantification of the compounds.

By extracting the organic layer with 0.1 *M* phosphate buffer, the relatively polar, phenolic metabolites 7-desmethyl and 8-desmethyl SCH-12679 were separated from SCH-12679 and its less polar, 3-desmethyl, derivative. Thus, washing of the toluene extract with phosphate buffer (pH 7.2) selectively removed the phenolic metabolites. It was essential to use the phosphate buffer as the recovery of 7-desmethyl and 8-desmethyl SCH-12679 was substantially lower when these compounds were not separated before the back-extraction cleanup step.

Measurement of SCH-12679 and its major metabolites

SCH-12679 was directly estimated with FID. 3-desmethyl SCH-12679 as well as 7- and 8-desmethyl SCH-12679 were acylated with HFBA and the derivatives were determined by ECD.

The residue left after extraction was dissolved in 25 μl of methanol by vortexing for 1 min, and 3 μl of this solution were injected into the gas chromatograph using FID.

For the estimation of metabolites only 10 μl of the rest of the solution were evaporated to dryness as described in the extraction procedure. The residue was dissolved in 50 μl of toluene. To this solution were added 20 μl of 0.1 *M* triethylamine in toluene followed by 5 μl of HFBA. The mixture was vibrated with a Vortex mixer for 1 min and kept at room temperature for 10 min. Then 100 μl of phosphate buffer (pH 6.0) were added to the solution. The mixture was vortexed as before and centrifuged for 10 min. About 2 μl of the toluene phase were injected into the gas chromatograph using ECD.

Calibration curves

The calibration curves were established using the internal standardization method. The methanolic solutions of the maleate salts of SCH-12679, 3-desmethyl SCH-12679, 7-desmethyl and 8-desmethyl SCH-12679 in the range 0.750—7.500 μg, 0.050—0.400 μg, 0.025—0.125 μg and 0.025—0.125 μg, respectively, were added to 3 ml of fresh heparinized plasma and the extraction was carried out as described above. Quantitation was achieved using the ratio of the peak areas of the compounds to that of internal standard protriptyline. Peak area ratios were plotted against weight ratios to obtain the calibration curves. The response of the FID to SCH-12679 was linear from 0.250 to 2.50 μg/ml of plasma. The ECD response to 3-desmethyl SCH-12679 and to 7-desmethyl plus 8-desmethyl SCH-12679 was linear over the range 0.017—0.133 and 0.008—0.042 μg/ml of plasma, respectively.

Human studies

Fifteen out of forty-one hospitalized aggressive mental retardates participating in a large-scale double-blind clinical study of SCH-12679 vs. placebo and thioridazine were the subjects of this experiment. The patients received a total of 400 mg of SCH-12679 per day orally in four doses at 8.00 a.m., 12.00 p.m., 4.00 p.m. and 8.00 p.m. for a period of four weeks. Two 10-ml

blood samples were drawn 2 h after the first morning dose at the end of the fourth week of treatment. Blood samples were centrifuged immediately after collection. An equal volume of plasma was aspirated in two separate tubes, which were wrapped in aluminium foil and placed in a deep-freeze at −15° until analysis.

RESULTS AND DISCUSSION

Selectivity

Analytical studies indicate that extracts from blank human plasma do not show peaks that could interfere with the quantitative analysis of SCH-12679 and its major metabolites. Typical chromatograms are shown in Figs. 2 and 3. The stationary phase, 3% PC-3210 (50% SE-30 ultraphase + 50% OV-210), gave a good resolution for SCH-12679, 3-desmethyl SCH-12679 and the internal standard, protriptyline. Although 3-desmethyl SCH-12679 could be identified by FID without derivatization with HFBA, the detector response to this compound was very low (Figs. 2 and 3). The isomers 7-desmethyl and 8-desmethyl SCH-12679 were not eluted from the PC-3210 column using similar conditions.

With the exception of SCH-12679 all its three major metabolites reacted with HFBA forming the corresponding derivatives (Fig. 3). The following

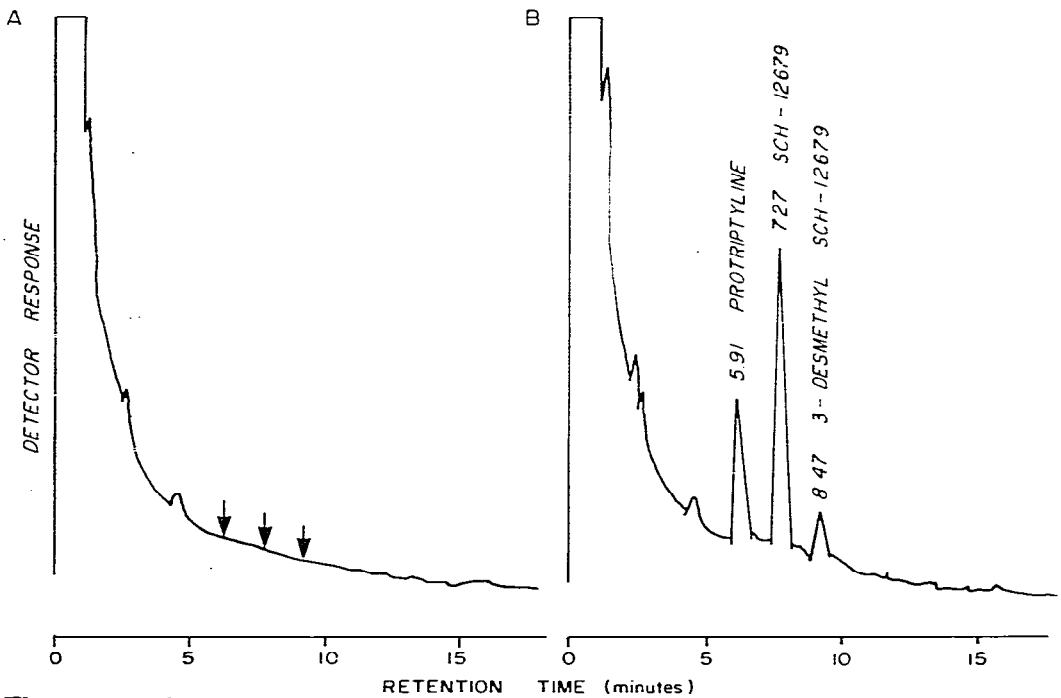

Fig. 2. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma. The arrows show the absence of signals at the retention times of the internal standard protriptyline, SCH-12679 and 3-desmethyl SCH-12679. (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the internal standard protriptyline, SCH-12679 and 3-desmethyl SCH-12679.

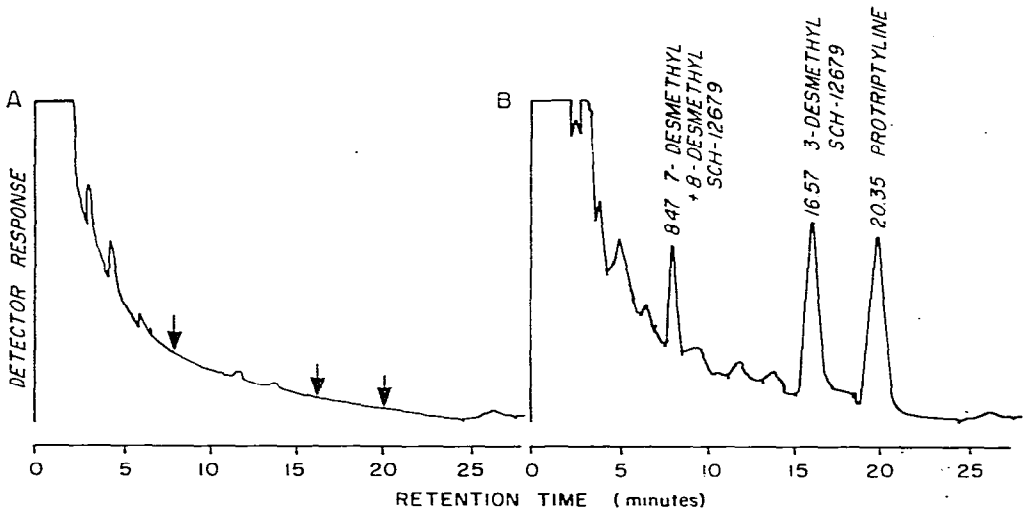

Fig. 3. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma derivatized with HFBA. The arrows show the absence of signals at the retention times of 7-desmethyl plus 8-desmethyl SCH-12679, 3-desmethyl SCH-12679 and the internal standard protriptyline. (B) Chromatogram obtained from an extract of 3 ml of human plasma containing 7-desmethyl plus 8-desmethyl SCH-12679, 3-desmethyl SCH-12679 and the internal standard protriptyline.

stationary phases were tried to resolve 7-desmethyl and 8-desmethyl SCH-12679: OV-7, OV-17, OV-210, SE-52, Dexsil 300, QF-1, Carbowax 20M, PC-3210 and Versamid 900. None of these phases could separate the two isomers. Apiezon L was selected as the stationary phase as it gave shorter retention times for the compounds assayed. Since all patients were kept under rigorous control as to the drug regimen during the four-week treatment period, the interference of other drugs and their metabolites was eliminated.

Recovery studies

The recoveries of SCH-12679, 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 from 3 ml of spiked plasma were determined using the same internal standardization method as described previously. The peak area ratio of each compound and the internal standard protriptyline was used as the index of detector performance and overall efficiency of the analytical procedure. The reproducibility and recovery results of SCH-12679 and its major metabolites are given in Table I. The overall coefficient of variation is below 10%.

Sensitivity

The lower limits for accurate determination of SCH-12679 and its metabolites were established by spiking 3 ml of blank plasma with dilute methanolic solutions of SCH-12679, 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 in the range of 60—600 ng, 1.5—50 ng, 1.2—25 ng and 1.2—25 ng, respectively, followed by the previously described extraction procedure using protriptyline as an internal standard. The lower detection limit was fixed to the minimum response of FID and ECD to the respective com-

TABLE I

GLC ESTIMATION OF SCH-12679, 3-DESMETHYL SCH-12679 AND A COMBINATION OF 7-DESMETHYL AND 8-DESMETHYL SCH-12679 ADDED TO PLASMA

Compound	Compound added (ng)	Compound recovered (ng)	Recovery* (%)	C.V. (%)
SCH-12679	67.7	63.8	94.2	4.8
	135.5	120.7	89.1	2.9
	270.9	251.4	92.8	3.5
	485.5	421.9	86.9	1.1
	677.3	591.3	87.3	2.3
3-Desmethyl SCH-12679	0.81	0.73	90.1	6.8
	2.43	2.08	85.6	2.5
	6.48	6.07	93.7	3.7
	9.70	8.61	88.8	5.1
	16.20	13.60	83.9	4.3
7-Desmethyl plus 8-desmethyl SCH-12679	0.405	0.292	72.0	9.7
	1.21	0.811	67.0	4.1
	2.00	1.55	77.5	6.8
	4.00	2.70	67.5	3.4
	6.00	4.16	69.3	5.2

*Each value is the mean of four determinations.

pounds with peak areas up to 40000 counts at an attenuation of × 128. It was found that the lowest detection limit of SCH-12679 with FID was 20 ng/ml of plasma and the ECD response to 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 was limited to 0.5 and 0.4 ng/ml of plasma, respectively.

Application of the method to human studies

The plasma levels of unchanged SCH-12679 and its major metabolites in psychiatric patients are presented in Table II. The plasma concentrations of SCH-12679 and its major metabolites measured in patients after four weeks of treatment show a marked individual variation. The plasma concentrations of SCH-12679 varied from 61 to 875 ng/ml, those of 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 from 34 to 534 ng/ml and 12 to 222 ng/ml, respectively. The interpatient variations in the steady-state plasma levels of antidepressants and neuroleptics in patients treated with these drugs had already been observed in earlier reports [9—14]. The therapeutic levels of SCH-12679 and its metabolites are not yet well defined. Of the three metabolites, only 8-desmethyl SCH-12679 has a significant behavioral activity in animals, whereas its isomer 7-desmethyl SCH-12679 is relatively inactive [15]. As 7-desmethyl and 8-desmethyl SCH-12679 could not be separated under the GLC conditions used, the two isomers were quantitated together in the ratio of 1:1. When the two isomers were analyzed separately, the ECD response to the heptafluorobutyryl derivative of each compound was different. If the two isomers were mixed in the ratio of 1:1, the resulting peak area was the sum of each heptafluorobutyryl derivative of the metabolites. This fact

TABLE II

PLASMA LEVELS OF SCH-12679, 3-DESMETHYL SCH-12679 AND A COMBINATION OF 7-DESMETHYL AND 8-DESMETHYL SCH-12679 IN PATIENTS RECEIVING SCH-12679 THERAPY

Patient	Sex	Age (years)	Weight (kg)	Plasma level of SCH-12679 (ng/ml)	Plasma level of 3-desmethyl SCH-12679 (ng/ml)	Plasma level of 7-desmethyl + 8-desmethyl SCH-12679 (ng/ml)
1	F	40	60	143	172	210
2	M	27	48	97	372	47
3	F	31	40	528	216	168
4	F	26	53	69	47	67
5	M	30	50	144	401	44
6	M	36	55	478	142	222
7	F	31	45	319	191	39
8	F	30	42	194	213	25
9	M	33	61	506	95	26
10	M	29	52	200	99	57
11	M	29	54	208	34	65
12	F	22	50	61	88	59
13	M	21	45	306	177	52
14	M	50	43	875	534	53
15	M	23	41	131	458	12

showed that the derivatization of the isomers with HFBA in the mixed ratio of 1:1 was complete and comparable to the separate analysis of each heptafluorobutyryl derivative of the respective metabolites.

To our knowledge, the present method is the first report for the assay of SCH-12679 and its major metabolites in human plasma. It can be useful in bioavailability studies in humans as well as in clinical and toxicological monitoring of patients.

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